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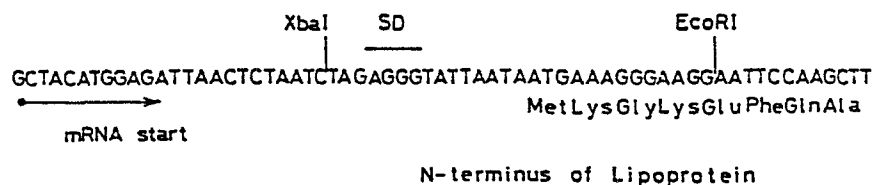
54 Improved plasmid vector and use thereof.

57 *Escherichia coli* plasmid vectors characterized in that they have a 5'-terminal untranslated region (inclusive of the propoter region and Shine-Dargano sequence) of the *Escherichia coli* lipoprotein gene, which region is improved to thereby enable direct production of useful polypeptides in substantially complete form.

FIG.1

pINI-A2

CCATCAAAAAATATTCTCAACATAAAAACTTTGTGTAATACTTGTAAC



Improved plasmid vector and use thereof

This invention relates to Escherichia coli plasmid vectors improved in that they are suited for the production of useful polypeptides and to the use thereof. More particularly, it relates to improved
5 plasmid vectors having a 5'-terminal untranslated region of the Escherichia coli lipoprotein gene, which region is improved in that a gene coding for a useful polypeptide can be inserted in an easy and simple manner and that said useful polypeptide can be produced
10 in substantially complete form, and to the production of useful polypeptides using Escherichia coli transformants transformed with said plasmids.

The so-called recombinant DNA technology (hereinafter abbreviated as rDNA technology) has so far
15 made it possible to produce a variety of useful polypeptides by the use of microbial cells or higher animal cells and, accordingly, it may be said that general techniques thereof have already been established. However, with respect to the technology
20 and method of economical production of each individual polypeptide, there still remains room for improvement.

In producing a certain useful polypeptide by utilizing rDNA technology, various factors exert influences on the productivity. It is known that,
25 among the factors the promoter involved in the promotion of transcription of an exogeneous gene coding

for the useful polypeptide as inserted and the
so-called Shine-Dalgarno sequence (SD sequence)
involved in complementary binding of mRNA to the
3'-terminal sequence of 16S ribosome RNA have a great
5 influence on the production of said useful peptide.
However, there is no rule established between the
promoter region or SD sequence and the productivity of
each individual polypeptide. Thus, for instance, the
fact that a certain promoter or SD sequence causes high
10 expression of a certain heterologous gene does not
always mean that it also allows high expression of
another heterologous gene.

Whereas the presence of a 3'-terminal untranslated
region of said gene is also a factor bringing about
15 high expression of an exogeneous gene and is important
especially for the expression of a heterologous gene in
eukaryotic cells, mention thereof is not made herein
because the invention has no direct relation thereto.
It is also known that some host cells are suited for
20 the expression of an objective gene but others are not
suited. No further mention is made in that respect,
either.

For the expression of an inserted heterologous
gene, various promoters are used depending on the host
25 cells.

When the host is Escherichia coli, for instance,
use is made of the Escherichia coli tryptophan

synthesis gene promoter, β -galactosidase gene promoter,
 β -lactamase gene promoter, alkaline phosphatase gene
promoter and lipoprotein promoter and the lambda (λ)
phage PL promoter, among others. Roughly speaking,
5 heterologous polypeptides are produced by two methods.
In the first method, an objective peptide is produced
as a hybrid protein with a certain protein while, in
the second method, the objective polypeptide is
produced directly, with a methionine residue added to
10 the N-terminus in some cases. Thus, for instance, a
hybrid protein from an objective peptide and a certain
protein connected with each other through methionine is
produced and then treated with cyanogen bromide (CNBr)
for cleaving the methionine residue, and the resulting
15 objective peptide is isolated. In that case, use of
the β -galactosidase gene promoter for producing an
objective peptide as a hybrid protein with alkaline
phosphatase protein is favorable in respect of
productivity of the objective peptide and purification
20 thereof (Japanese Patent Application No. 107,474/1982).
For producing polypeptides, such as interferons, by
direct expression, there are used, for example, the
tryptophan gene promoter, β -galactosidase gene promoter

and λ phage PL promoter (cf., for example, P.W. Gray et al., Nature, 295: 503, 1982; D. Goeddel et al., Nature, 287: 411, 1980; T. Taniguchi et al., Proc. Natl. Acad. Sci., 77: 5230, 1980; R. Derynck et al., Nature, 287:
5 193, 1980).

On the other hand, one of the factors required for efficient production of a heterologous polypeptide in Escherichia coli is the SD sequence which is upstream from the translation initiation codon (ATG). The
10 complementarity of this SD sequence and the 3'-terminal sequence of 16S ribosome RNA, the distance between the SD sequence and translation start codon, and the secondary structure of mRNA, at and around the translation startpoint, among others, are supposed to contribute to
15 efficient initiation of translation. It is therefore presumable that the productivity of the polypeptide to be produced depends on the amino acid sequence of that portion of said peptide which is close to the N-terminus, or, in other words, the DNA sequence corresponding to
20 said portion.

Paying their attention to the above points, the present inventors modified the nucleotide sequence of the 5'-terminal untranslated region of the Escherichia

coli lipoprotein gene in various ways and thereby constructed Escherichia coli plasmids into which exogeneous genes can be inserted for very efficient expression thereof and which allow efficient production of objective polypeptides in substantially complete form, and have now completed the present invention. Furthermore, the use of the plasmid vectors according to the invention led to efficient production of a polypeptide having the amino acid sequence of human immune interferon (hereinafter abbreviated as hINF- γ), whereby culture compositions containing said polypeptide in high concentrations could be obtained.

Thus, the present invention provides Escherichia coli plasmid vectors characterized in that they have a 5'-terminal untranslated region (inclusive of the promoter region and Shine-Dalgarno sequence) of the Escherichia coli lipoprotein gene, which region is improved to thereby enable direct production of useful polypeptides in substantially complete form.

The Escherichia coli lipoprotein is a protein which constitutes the outer membrane of Escherichia coli, the content being 4.8×10^5 molecules per cell (Bacterial Outermembrane, Biogenesis and Functions,

edited by Masayori Inouye, page 8, Wiley-Interscience Publication, Toronto, 1979). It is also known that the corresponding lipoprotein gene has a promoter potent in transcription activity.

5 The gene covering the lipoprotein promoter region (5'-terminal untranslated region) to the structural gene for said protein has already been cloned in pBR322 and a series of plasmids modified to thereby enable expression of exogeneous genes inserted therein have
10 been constructed (cf. Kagaku to Seibutsu, 20, No. 1, 47-58, 1982). A patent specification laid open discloses heterologous polypeptide production using the above promoter region (Japanese Kokai Tokkyo Koho No. 140,800/1982).

15 However, the cloning vector disclosed in the above has serious drawbacks, such as mentioned below. The cloning site (site for insertion of exogeneous polypeptide-encoding genes; in said vector, the EcoRI cleavage site) of said vector is 10-11 bases downstream
20 from the translation startpoint and therefore the desired product polypeptides have, on the N-terminus side thereof, an extra peptide added thereto. This offers a problem of antigenicity upon administration to

the human body, even if the product polypeptides are physiologically active. Such polypeptides are never preferred since they have a structure different from the original one.

5 In accordance with the invention, novel high expression vectors free of the drawbacks that the above known vector has are constructed through modification of the SD sequence of the lipoprotein gene, modification of the distance between the SD sequence
10 and translation startpoint and insertion of the cloning site at a new site, among others. Typical examples have proved their utility.

Hereinbelow, the plasmid vectors according to the invention and use thereof are described in more detail.

15 The base sequence including the Escherichia coli lipoprotein promoter region and part (upstream portion) of the structural gene for said protein on pINIA2, which is one of the plasmids reconstructed to thereby enable expression of exogenous genes, is shown in Fig.
20 1. This plasmid has drawbacks such as mentioned above, since the cloning site (EcoRI in Fig. 1) for insertion of exogenous genes to be expressed is in the lipoprotein structural gene (in the DNA sequence coding

for the signal peptide). The base sequence from the promoter region to an upstream portion of the structural gene (to the EcoRI cleavage site) as shown in Fig. 1 is identical with that of one of the
5 expression vectors disclosed in the above-cited Japanese Kokai Tokkyo Koho No. 140,800/1982.

Accordingly, for avoiding extra peptide addition to the N-terminus of desired heterologous polypeptides, the present inventors replaced the XbaI-EcoRI fragment
10 shown in Fig. 1 with a chemically synthesized oligonucleotide fragment (having XbaI and EcoRI cohesive ends at the respective ends and containing the SD sequence), followed by connection of the gene coding for a desired useful polypeptide (for example,
15 polypeptide having the amino acid sequence of hIFN- γ) downstream from said EcoRI site. The thus-constructed plasmid pIN4GIF54 was used for transformation of Escherichia coli. The transformant (W3110/pIN4GIF54), under the control of the lipoprotein gene
20 promoter, could produce hIFN- γ without any extra peptide added to the N-terminus thereof.

In the above explanation, hIFN- γ has been given as an example of the heterologous useful polypeptide for

better understanding. It is obvious that the above principle can also be applied to the production of other polypeptides.

Fig. 2 schematically illustrates the construction of pIN4GIF54. In the figure, pGIF54 is essentially the same plasmid as pGIF4 disclosed in Japanese Patent Application No. 86,180/1982. An Escherichia coli transformant, WA802/pGIF4, obtained by transformation with said plasmid containing the chemically synthesized gene coding for the amino acid sequence of hIFN- γ as shown in Fig. 6 has been named SBMG105 and deposited with the Fermentation Research Institute, the Agency of Industrial Science and Technology [Deposit No.: FERM P-6522; International Deposit No. (under the Budapest Treaty): FERM BP-282]. The plasmid pINI-A2 is a gift from Mr. Inoue of New York State University. A host Escherichia coli strain obtained by transformation with said plasmid has been named JA221/PINIA2 and deposited with the Fermentation Research Institute under Deposit No. FERM BP-320.

Fig. 3 illustrates the nucleotide sequence from the 5'-terminal untranslated region of the lipoprotein gene as modified according to the invention to an

- 10 -

upstream portion of the hINF- γ gene inserted. The SD sequence shown in Fig. 3 is the same as that of the lipoprotein gene and the distance between the SD sequence and the translation initiation codon remains
 5 unaltered.

While the strain of Escherichia coli transformed with pIN4GIF54 constructed in the manner shown in Fig. 2, W3110/pIN4GIF54, also produced hINF- γ (as described later in detail), the inventors attempted further
 10 modification of the SD sequence for increasing the productivity (cf. Fig. 4). Thus, they designed the SD sequence oligonucleotide so as to make it complementary to the 3'-end sequence of Escherichia coli 16S ribosome RNA, and chemically synthesized the DNA fragment shown
 15 in Fig. 4, namely

C	T	A	G	G	A	G	G	T	A	G
				C	T	C	C	A	T	C
										T
										T
										A
										A

Then, pIN5GIF54 was constructed by replacing the XbaI-EcoRI fragment of pIN4GIF54 with the above DNA
 20 fragment. Escherichia coli transformed with this plasmid, W3110/pIN5GIF54, was found to produce hINF- γ in an amount 4 times greater as compared with the case of W3110/pIN4GIF54 (cf. Table 2), indicating that the

modification of the SD sequence according to the invention is effective. Furthermore, culture compositions containing hIFN- γ in high concentrations could be obtained from the cultured cells of said
5 transformant.

In the example of the invention, ampicillin-resistant gene is inserted as a drug-resistant gene into the plasmid of the invention (ref. Fig.s 2 and 4), however, the drug-resistant gene is not limited
10 to the example, and other known drug-resistant genes may be employed. For instance, well-known tetracycline- or kanamycin-resistant gene may be used instead of the ampicillin-resistant gene without injuring the essence of the invention.

15 The drawings are briefly explained as follows;

Fig. 1 shows the base sequence of the 5'-terminal untranslated region of the Escherichia coli lipoprotein gene, which includes the promoter region and SD
20 portion) of the structural gene for said protein.

Fig. 2 schematically illustrates the construction of the hIFN- γ gene expression plasmid vector pIN4GIF45.

Fig. 3 shows the base sequence covering the improved 5'-terminal untranslated region of the lipoprotein gene
25 on the plasmid pIN4GIF54. Fig. 4 schematically illustrates the construction of the hIFN- γ gene expression plasmid vector pIN5GIF54. Fig. 5 shows the

base sequence covering the improved 5'-terminal
untranslated region of the lipoprotein gene on the
plasmid pIN5GIF54. Fig. 6 shows the bases sequence of
a chemically synthesized hINF- γ gene and the
5 corresponding amino acid sequence. Fig. 7 and Fig.
8 schematically illustrate the construction of the
plasmids PIN5T4 and PIN5T5, respectively.

The following examples illustrate the invention in
more detail.

10 Examples

I. Construction of pIN4GIF54

The plasmid pIN4GIF54 was constructed, as shown in
Fig. 2, from (1) DNA fragment containing the
lipoprotein gene promoter region (indicated by lpp in
15 the figure) as obtained by digestion of the plasmid
pINIA2 with the restriction enzymes XbaI and PstI, (2)
oligonucleotide having XbaI and EcoRI cohesive ends and
(3) DNA fragment containing the hINF- γ gene as obtained
by digestion of the plasmid pGIF54 with EcoRI and PstI.
20 The procedure followed was as described hereinbelow.
The restriction enzymes used were all products of
Takara Shuzo KK.

A) Preparation of XbaI-PstI DNA fragment of pINIA2

The pINIA2 DNA (3 μ g) was digested with 15 units
25 each of XbaI and PstI in 150 μ l of 1 X TA solution (33

mM Tris acetate buffer, pH 7.6, 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM dithiothreitol) at 37°C for 60 minutes. The reaction mixture was subjected to 1.0% agar gel electrophoresis and a gel portion located at the position corresponding to about 980 b.p. (base pairs) was cut out and placed in a dialysis tube, and the XbaI-PstI DNA fragment was eluted by electrophoresis. After removal of ethidium bromide from the eluate by adding an equal amount of phenol thereto, 2.5 volumes of ethanol was added. After standing at -80°C for 30 minutes, the mixture was centrifuged at 10,000 rpm for 10 minutes, whereby the DNA fragment was obtained as an ethanol precipitate. To this ethanol precipitate was added 10 µl of distilled water for dissolving the DNA fragment.

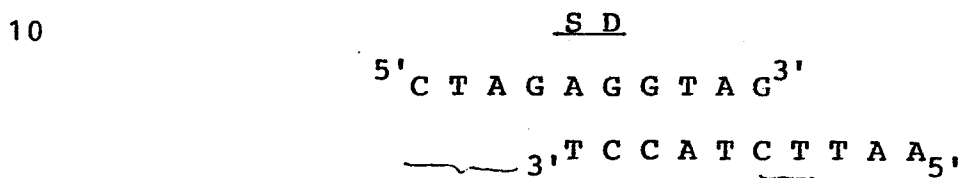
B) Preparation of EcoRI-PstI DNA fragment of pGIF54.

The pGIF54 DNA (3 µg) was digested with 15 units each of EcoRI and PstI in 30 µl of 1 X TA solution at 37°C for 60 minutes, followed by 0.7% agar gel electrophoresis, whereby an EcoRI-PstI DNA fragment of about 3.4 Kb was eluted from the gel. The eluate was subjected to phenol treatment and ethanol precipitation in the same manner as above. To the ethanol

precipitate, 10 μ l of distilled water was added for dissolution of the DNA fragment.

C) Preparation of oligonucleotide having XbaI and EcoRI cohesive ends.

5 For the expression of complete hINF- γ protein, an oligonucleotide having the Shine-Dalgarno (SD) sequence downstream from the XbaI cleavage site of pINIA2 and further having an EcoRI cohesive end, namely the oligonucleotide



XbaI cohesive end EcoRI cohesive end

15 was synthesized by the solid phase method. The synthetic procedure has been disclosed in detail in Japanese Patent Application No. 86,180/1982.

The above oligonucleotide (100 picomoles) was phosphorylated at the 5'-OH in 30 μ l of a kinase

20 reaction solution (50 mM Tris hydrochloride buffer, pH 8.0, 10 mM MgCl_2 , 10 mM dithiothreitol), with 2 units of T4 polynucleotide kinase (Takara Shuzo KK) added, at 37°C for 60 minutes.

D) Construction of pIN4GIF54

The plasmid pIN4GIF54 was constructed by ligation of the three DNA fragments prepared above in accordance with the following procedure. Thus, to a mixture of 5 μ l of a solution of the XbaI-PstI DNA fragment of pINIA2 (solution of the ethanol precipitate in 10 μ l of distilled water), 5 μ l of a solution of the EcoRI-PstI DNA fragment of pGIF54 (solution of the ethanol precipitate in 10 μ l of distilled water) and 3 μ l of a solution of the phosphorylated oligonucleotide (10 picomoles), there were added 2 μ l of a ligation reaction medium 10-fold higher in concentration (20 mM Tris hydrochloride buffer, pH 7.6, 10 mM $MgCl_2$), 2 μ l of 4 mM ATP and 1 μ l of a solution of T4 DNA ligase (Boehringer Mannheim) (5 units), and the ligation was carried out at 16°C overnight.

II. Transformation of Escherichia coliA) Transformation of Escherichia coli WA802

Escherichia coli WA802 was cultured in 2.0 ml of L-broth at 37°C overnight, 0.3 ml of the culture broth was added to 30 ml of L-broth, and shake culture was performed at 37°C for 2 hours, followed by centrifugation at 3,000 rpm for 10 minutes. To the

thus-obtained cells was added 10 ml of 50 mM CaCl_2 for suspending the cells, and centrifugation was conducted at 3,000 rpm for 10 minutes. To the thus-obtained cells was added 1.0 ml of 50 mM CaCl_2 solution, and the mixture was allowed to stand in an ice bath for 60 minutes. To 0.2 ml of this suspension of Ca^{++} -treated cells was added 10 μl of the ligation reaction mixture obtained in Example I-D (containing the above-mentioned three DNA fragments ligated), the mixture was allowed to stand in an ice bath for 60 minutes, then 2 ml of L-broth was added and incubation was conducted at 37°C for 60 minutes. The culture broth was used for plating a nutrient agar medium (BBL) containing 40 $\mu\text{g}/\text{ml}$ of ampicillin. After incubation at 37°C overnight, ampicillin-resistant transformants were selected. One of the transformants obtained was used for plasmid DNA separation therefrom by the conventional method (cleared lysate method). The base sequence of the DNA at and around the XbaI-EcoRI region inserted was determined by the Maxam-Gilbert method (Methods in Enzymology, 65: 499-560, 1980) and it was confirmed that the DNA had the desired DNA base sequence. This plasmid was named pIN4GIF54 and the

transformant Escherichia coli strain carrying the same was named WA802/pIN4GIF54.

The base sequence of pIN4GIF54 at and around the promoter is shown in Fig. 4. In this pIN4GIF54, the
5 Shine-Dalgarno (SD) sequence is the same as that in pINIA2 and the distance between the SD sequence and the translation initiation codon ATG for hIFN- γ is 9 b.p., which is the same as in pINIA2. Nevertheless, owing to the EcoRI cleavage site introduced between the SD
10 sequence and translation initiation codon ATG, modification of the SD sequence and of the distance between the SD sequence and translation initiation codon can be made with ease and therefore this vector is considered to be an advantageous vector for use in
15 constructing high expression vectors not only for the hIFN- γ gene but also for other genes.

B) Transformation of Escherichia coli W3110

The pIN4GIF54 plasmid obtained was used for transformation of Escherichia coli by the
20 above-mentioned method. Ampicillin-resistant strains were isolated and one of the transformants was named W3110/pIN4GIF54. This transformant was tested for antiviral activity.

III. Test of transformant W3110/pIN4GIF54 for
antiviral activity

For examining the amount of hIFN- γ expressed,
W3110/pIN4GIF54 and, for comparison, W3110/pGIF54
5 (essentially the same strain as the transformant
disclosed in Japanese Patent Application No.
86,180/1982 and deposited with the Fermentation
Research Institute under Deposit No. 6522) were
cultivated in the manner mentioned below and the
10 culture supernatants were assayed for antiviral
activity. The host strain W3110 was used as a control.

The strains W3110/pIN4GIF54 and W3110 were
cultivated in 2.0 ml of L-broth containing 40 μ g/ml of
ampicillin at 37°C overnight, 0.1 ml of the culture
15 broth was used for inoculation of 5 ml of L-broth, and
incubation was performed at 37°C for 4 hours. With
W3110/pGIF54, 0.1 ml of a preculture broth was used for
inoculation of 5 ml of the same L-broth as above,
incubation was conducted at 37°C for 2 hours, then
20 isopropyl- β -D-thiogalactopyranoside (hereinafter
abbreviated as IPTG) was added in a final concentration
of 1 mM and the incubation was continued for further 2
hours.

Cells were harvested by centrifuging 4 ml of each culture broth at 3,000 rpm for 15 minutes. To the cells obtained, there was added 200 μ l of 0.15 M sodium phosphate buffer, 50 mM NaCl, pH 7.2, containing 1
5 mg/ml of lysozyme (Sigma, USA) (hereinafter abbreviated as 1 X PBS). The mixture was allowed to stand on an ice bath for 30 minutes to thereby effect bacteriolysis. Thereafter, the lysate was frozen quickly with dry ice-methanol and then thawed quickly
10 in a constant-temperature bath maintained at 37°C. After disintegration of the cells by three repetitions of such freezing and thawing treatment, the lysate was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was assayed for antiviral activity by the
15 method described in Japanese Patent Application No. 86,180/1982.

The results thus obtained are shown in Table 1.

Table 1

Antiviral activity of transformants

20	<u>Strain</u>	<u>Antiviral activity I.u./ml</u>
	W3110	0
	W3110/pGIF54	400
	W3110/pIN4GIF54	1000

The antiviral activity was completely lost at pH 2. Neutralization with a hIFN- γ -specific antibody also resulted in complete inactivation, whereas antibodies specific to α -type and β -type interferons practically
5 failed to cause inactivation.

As seen in Table 1, W3110/pIN4GIF54 showed 2.5 times higher antiviral activity as compared with W3110/pGIF54. However, the degree of expression cannot yet be said to be satisfactory. Therefore, the
10 lipoprotein SD sequence was modified. The lipoprotein SD sequence is GAGG (4 bp) and this sequence is complementary to the sequence CUCC (indicated below by $\bullet\bullet\bullet\bullet$) of the 3'-terminal 3' $\overline{\text{AUUCCUCCAG}}$ 5' of Escherichia coli 16S ribosome RNA.

15 The inventors expected that conversion of the lipoprotein SD sequence complementary to said 16S ribosome RNA to AGGAGGT which is used in the R17A or MS2A protein SD sequence might result in an increase in complementarity with 16S ribosome RNA, hence in
20 translation activity, so that the expression of the hIFN- γ protein could be increased.

Therefore, they attempted to insert the SD sequence AGGAGGT between the XbaI-EcoRI sites of the

plasmid pIN4GIF54. For that purpose, an oligonucleotide having the above sequence as well as XbaI and EcoRI cohesive ends at the 5'-ends, namely

C T A G G A G G T A G

C T C C A T C T T A A

5 and this was inserted between XbaI-EcoRI of pIN4GIF54, whereby the plasmid pIN5GIF54 was constructed (cf. Fig. 4).

The following are the details.

10 IV. Construction of pIN5GIF54 plasmid (cf. Fig. 4)

A) Preparation of oligonucleotide having XbaI and EcoRI cohesive ends

The oligonucleotide having the SD sequence AGGAGGT and XbaI and EcoRI cohesive ends at the 5'-ends, namely

15 5' C T A G G A G G T A G 3'

3' C T C C A T C T T A A 5'

was synthesized by the solid phase method mentioned above (cf. Japanese Patent Application No.

86,180/1982). The above oligonucleotide (100 picomoles) was phosphorylated at the 5'-OH, at 37°C for
20 60 minutes, in 50 µl of the kinase reaction solution (detailedly described in Example I-C) with 2 units of

T4 polynucleotide kinase (Takara Shuzo) added, as mentioned above.

B) Preparation of XbaI-EcoRI DNA fragment of pIN4GIF54

pIN4GIF54 (2.5 µg) was digested with 5 units each
5 of XbaI and EcoRI in 30 µl of 1 X TA solution at 37°C
for 60 minutes for cleaving the DNA. After cleavage,
0.7% agar gel electrophoresis was carried out and an
XbaI-EcoRI DNA fragment of about 4.3 Kb (SD sequence-
free longer fragment) was eluted from the gel by
10 electrophoresis as mentioned above. The eluate was
subjected to phenol treatment and ethanol precipitation
as mentioned above and, to the ethanol precipitate, 10
µl of distilled water was added for dissolution of the
DNA fragment.

15 C) Construction of pIN5GIF54

The plasmid pIN5GIF54 was constructed by ligating
the above two DNA fragments in the following manner.
Thus, to 5 µl a solution of the phosphorylated
oligonucleotide (10 picomoles), there were added 3 µl
20 of the ligation reaction solution 10-fold in
concentration (mentioned hereinbefore), 10 µl of 100 mM
DTT and 4 mM ATP in distilled water, and 1 µl (5 units)

of T4 DNA ligase (Boehringer Mannheim). The mixture was incubated at 16°C overnight.

V. Transformation of Escherichia coli

A) Transformation of Escherichia coli WA802

5 In the same manner as above (Example II-A), cells of Escherichia coli WA802 as grown in L-broth were treated with CaCl_2 , and 0.2 ml of cell suspension was mixed with the ligation reaction mixture obtained in Example IV-C for effecting transformation of

10 Escherichia coli WA802. Transformant selection was conducted using a nutrient agar medium (BBL) containing 40 µg/ml of ampicillin. Using one of the transformant strains thus obtained, plasmid separation was performed by the conventional method (cf. Example II-A), and the

15 DNA base sequence at and around the insert region, namely XbaI-EcoRI region, was analyzed. As shown in Fig. 5, pIN5GIF54 must be free of the XbaI cleavage site originally present in pIN4GIF54 (fig. 3).

Therefore, the plasmid separated was treated with XbaI,

20 followed by 0.7% agar gel electrophoresis and determination, for the plasmid DNA remaining uncleaved with XbaI, of the DNA sequence at and around the oligonucleotide fragment insert by the Maxam-Gilbert method. The results obtained confirmed the presence of

the intended DNA base sequence. This plasmid was named pIN5GIF54, and the WA802 strain transformed therewith was named WA802/pIN5GIF54.

B) Transformation of Escherichia coli W3110

5 The pIN5GIF54 plasmid obtained above was used for transformation of Escherichia coli W3110 in the same manner as above. An ampicillin-resistant transformant was isolated and named W3110/pIN5GIF54. This transformant was compared with W3110/pIN4GIF54 with
10 respect to antiviral activity.

VI. Test of W3110/pIN4GIF54 and W3110/pIN5GIF54 for
antiviral activity

W3110/pIN4GIF54, W3110/pIN5GIF54 and, as a control, W3110 were each grown in 2.0 ml of L-broth
15 (containing 40 µg/ml of ampicillin) at 37°C overnight, and 0.1 ml of each culture broth was used for inoculating 5 ml of L-broth (containing 40 µg/ml of ampicillin), followed by further cultivation for 2 hours. A 2-ml portion of each culture broth was
20 centrifuged at 3,000 rpm for 15 minutes. To the cells thus obtained was added 1.0 ml of 1 X PBS containing 1 mg/ml of lysozyme, and the mixture was allowed to stand

for 30 minutes to effect bacteriolysis. Thereafter, the cells were disintegrated by three respective repetitions of rapid freezing with dry ice-methanol and rapid thawing at 37°C, and the supernatant obtained by
5 centrifugation at 10,000 rpm for 10 minutes was assayed for antiviral activity by the method described in Japanese Patent Application No. 86,180/1982. The results thus obtained are shown in Table 2.

Table 2

10 Antiviral activity measurement

<u>Strain</u>	<u>I.u./ml</u>
W3110	0
W3110/pIN4GIF54	1000
W3110/pIN5GIF54	4000

15 As is evident from Table 2, W3110/pIN5GIF54 exhibited 4 times higher antiviral activity as compared with W3110/pIN4GIF54, indicating that the improvement in the SD sequence had been effective in increasing the expression yield of hIFN- γ protein.

20 Escherichia coli W311D transformed by plasmids PIN5T4 and PIN5T5 wherein tetracycline-resistant gene (Tc^r) is inserted instead of ampicillin-resistant gene (Ap^r) on PIN5GIF54 exhibited the same antiviral activity as W3110/PIN5GIF54 mentioned above.

PIN5T4 was prepared by the ligation of DNA fragment containing hIFN- γ gene and DNA fragment containing Tc^r gene with T4DNA ligase after making the cohesive ends to smooth ends with T4DNA polymerase and dNTP.

5 The former DNA fragment was obtained by cleaving PIN5-GIF54 with restriction enzymes AatII and SalI, and the latter by cleaving pBR322 with EcoRI and AhaIII (Fig. 7).

10 PIN5T5 was able to be prepared by the same procedure as above except for cleaving pBR322 with EcoRI and PstI (Fig. 8).

The antiviral activity disappeared upon treatment at pH 2 and upon neutralization with a hIFN- γ -specific antibody. These facts clearly indicate that these
15 transformants are hIFN- γ -producing strains.

The dates of deposit of the microorganisms referred to above are as follows:-

	<u>FERM NO.</u>	<u>Deposit Date</u>
	P-6522	May 6th, 1982
20	BP-282	May 2nd, 1983
	BP-320	July 18th, 1983.

Claims:

(1) An Escherichia coli plasmid vector which contains a 5'-terminal untranslated region (inclusive of the promoter region and Shine-Dalgarno sequence) of the Escherichia coli lipoprotein gene, said region
5 being improved in that it enables direct production of a useful polypeptide in substantially complete form.

(2) A plasmid vector as set forth in Claim 1, which contains a sequence improved in that it enables stable and complementary binding of the Shine-Dalgarno
10 sequence to the 3'-terminus of Escherichia coli 16S ribosome RNA.

(3) A plasmid vector as set forth in Claim 1 or 2 which contains an appropriate restriction enzyme cleavage sequence between the Shine-Dalgarno sequence
15 and the translation initiation codon ATG.

(4) A plasma vector as set forth in Claim 3, wherein the restriction enzyme cleavage site is a sequence cleavable with EcoRI.

(5) A plasmid vector as set forth in Claim 2,
20 wherein the Shine-Dalgarno sequence contains the sequence, 5' AGGAGGT 3'.

(6) A plasmid vector as set forth in Claim 1, wherein a gene coding for the useful polypeptide is inserted downstream from the 5'-terminal untranslated
25 region (inclusive of the promoter region and Shine-Dalgarno sequence).

(7) A plasmid vector as set forth in Claim 6, wherein the useful peptide has the amino acid sequence of human immune interferon.

(8) A plasmid vector as set forth in Claim 7,
5 wherein the gene coding for the amino acid sequence of human immune interferon is a chemically synthesized gene or a gene produced with the aid of purified immune interferon mRNA from human pancreatic tissues or peripheral blood lymphocytes.

10 (9) A plasmid vector as set forth in Claim 8, wherein the chemically synthesized gene contains the base sequence as shown in Fig. 6.

(10) A plasmid vector as set forth in Claim 1, wherein the plasmid is selected from the group
15 consisting of pIN4GIF54 and pIN5GIF54.

(11) A plasmid vector as set forth in Claim 1 which contains the base sequence as shown in Fig. 3.

(12) A plasmid vector as set forth in Claim 5 which contains the base sequence as shown in Fig. 5.

20 (13) An Escherichia coli transformant obtained by transformation with a plasmid as set forth in any of Claims 7-12.

(14) A transformant as set forth in Claim 13 which is selected from the group consisting of
25 WA802/pIN4GIF54, WA802/pIN5GIF54, W3110/pIN4GIF54 and W3110/pIN5GIF54.

(15) A culture containing a polypeptide having the amino acid sequence of human immune interferon, said culture being obtained by cultivating a transformant as set forth in Claim 13 or 14.

FIG.1

pIN1-A2

CCATCAAAAAATATTCTCAACATAAAAAAACTTTGTGTAATACTTGTAAC

XbaI
SD
EcoRI
|
|
|
GCTACATGGAGATTAACTCTAATCTAGAGGGTATTAAATGAAAGGGAAGGAATTCCAAGCTT
→
→
|
mRNA start
MetLysGlyLysGluPheGlnAla

N-terminus of Lipoprotein

FIG.2

Construction of pIN4GIF54 Plasmid

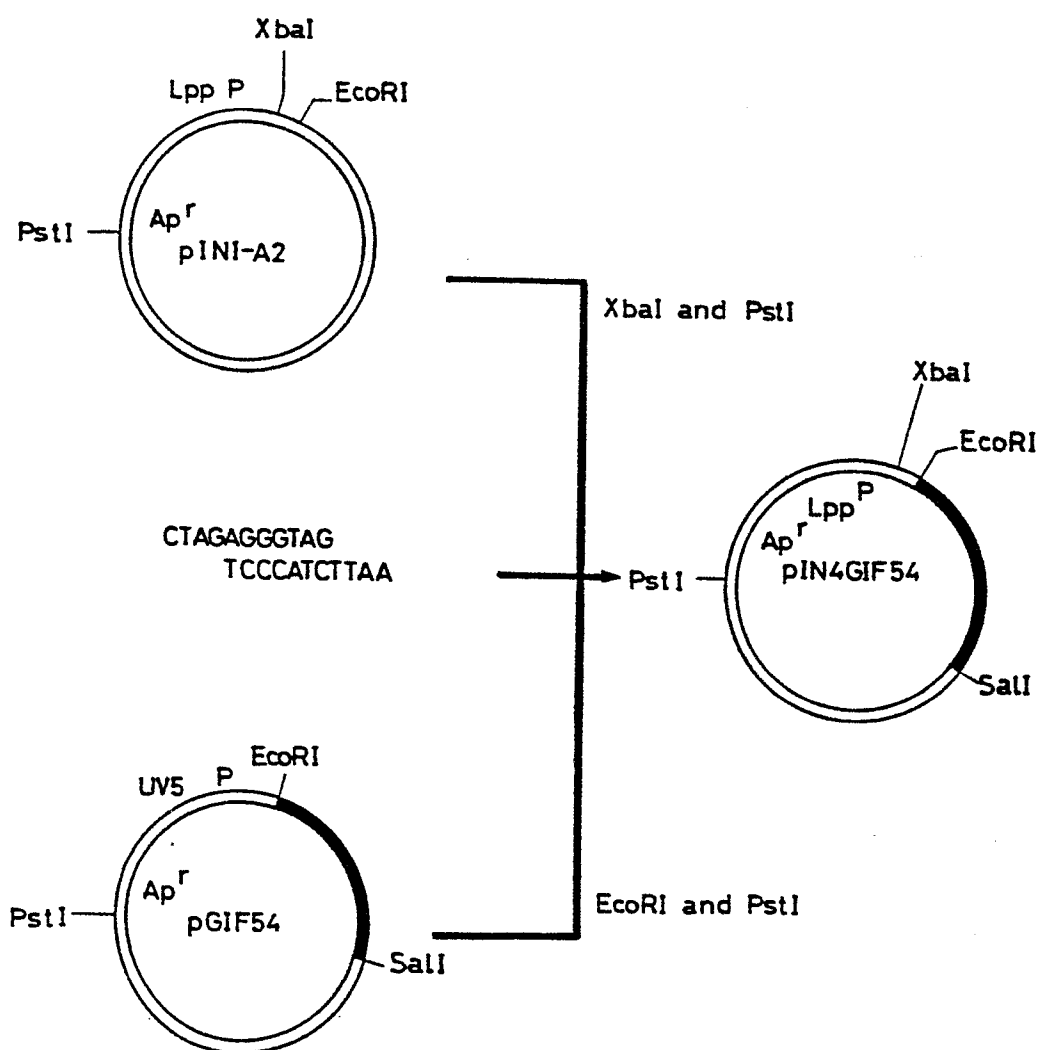


FIG.3

pIN4GIF54

CCATCAAAAAAATATTCTCAACATAAAAACTTTGTGTAATACTTGTAAC

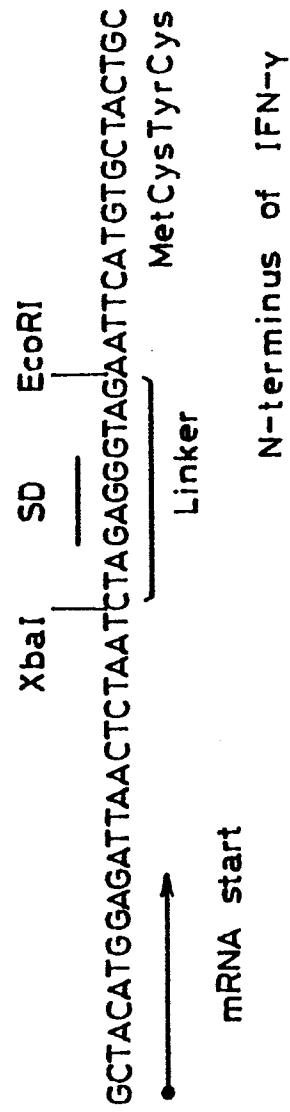


FIG. 4

Construction of pIN5GIF54 Plasmid

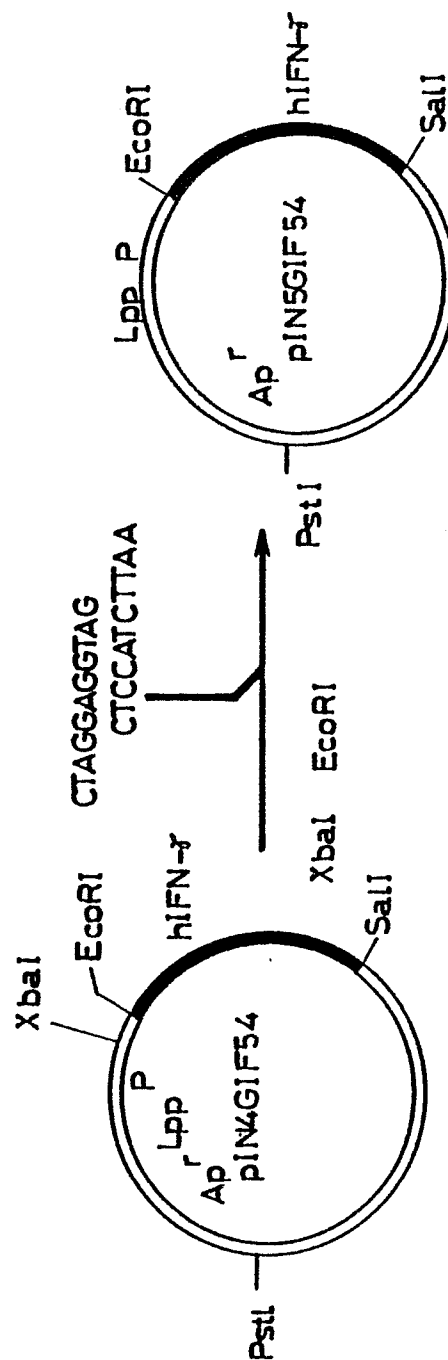


FIG.5

pIN5GIF54

CCATCAAAAAAATATTCTCAACATAAAAACTTTGTGTAATACTTGTAAC

SD EcoRI

GCTACATGGAGATTAACTCTAATCTAGGAGGTAGAAATTCATGTGCTACTGC

MetCysTyrCys

Linker

N-terminus of IFN-γ

mRNA start

FIG.6

1 Met Cys Tyr Cys Gln Asp Pro Tyr Val Lys Glu Ala Glu Asn Leu Lys Lys Tyr Phe Asn Ala Gly His Ser Asp
 5' AATTC ATG TGC TAC TGC CAG GAC CCA TAC TAC GGT AAG GAA GCT GAA AAC CTG AAG AAA TAC TTC AAC GCT CAT TCT GAC
 3' G TAC ACG ATG ACG GTC CTG GGT ATG CAC TTC CTT CGA CTT TTG GAC TTC TTT ATG AAG TTG CGA CCA GTA AGA CTG
 20
 Val Ala Asp Asn Gly Thr Leu Phe Leu Gly Ile Leu Lys Asn Trp Lys Glu Ser Asp Arg Lys Ile Met Gln
 GTT GCT GAC AAC GGT ACT CTG TTC CTG GGT ATC CTG AAA AAC TGG AAA GAA GAA TCT GAC CGT AAA ATC ATG CAG
 CAA CGA CTG TTG CCA TGA GAC AAG GAC CCA TAG GAC TTT TTG ACC TTT CTT CTT AGA CTG GCA TTT TAG TAC GTC
 40
 50 Gln Ile Val Ser Phe Tyr Phe Lys Leu Phe Lys Asn Phe Lys Asp Asp Gln Ser Ile Gln Lys Ser Val Glu
 Ser TCT CAG ATC GTT TCT TTC TAC TTC AAG CTG TTC AAA AAC TTC AAG GAC GAC CAG TCT ATC CAG AAA TCT GTT GAA
 AGA GTC TAG CAA AGA AAG ATG AAG TTC GAC AAG TTT TTG AAG TTC CTG CTG GTC AGA TAG GTC TTT AGA CAA CTT
 60
 80 Thr Ile Lys Glu Asp Met Asn Val Lys Phe Phe Asn Ser Asn Lys Lys Lys Arg Asp Asp Phe Glu Lys Leu Thr
 ACT ATC AAG GAA GAC ATG AAC GTT AAG TTC TTC AAC TCT AAC AAG AAA AAG CGT GAC GAC TTC GAA AAG CTT ACT
 TGA TAG TTC CTT CTG TAC TTG CAA TTC AAG AAG TTG AGA TTG TTC GCA CTG CTG AAG CTT TTC GAA TGA
 90
 100 Asn Tyr Ser Val Thr Asp Leu Asn Val Gln Arg Lys Ala Ile His Glu Leu Ile Gln Val Met Ala Glu Leu Ser
 AAC TAC TCT GTT ACT GAC CTT AAT GTA CAG CGT AAA GCT ATC CAT GAA CTG ATC CAG GTT ATG GCT GAA CTG TCC
 TTG ATG AGA CAA TGA CTG GAA TTA CAT GTC GCA TTT CGA TAG GTA CTT GAC TAG GTC CAA TAC CGA CTT GAC AGG
 110
 130 Pro Ala Ala Lys Thr Gly Lys Arg Lys Arg Ser Gln Met Leu Phe Arg Arg Ala Ser Gln Slp
 CCG GCT GCT AAA ACT GGT AAG CGT AAA AGA TCT CAG ATG CTG TTC CGT GGT CGT GCT TCT CAG TAA G 3'
 GGC CGA CGA TTT TGA CCA TTC GCA TTT TCT AGA GTC TAC GAC AAG GCA CCA GCA GCA AGA GTC ATT CAGCT 5'

FIG.7

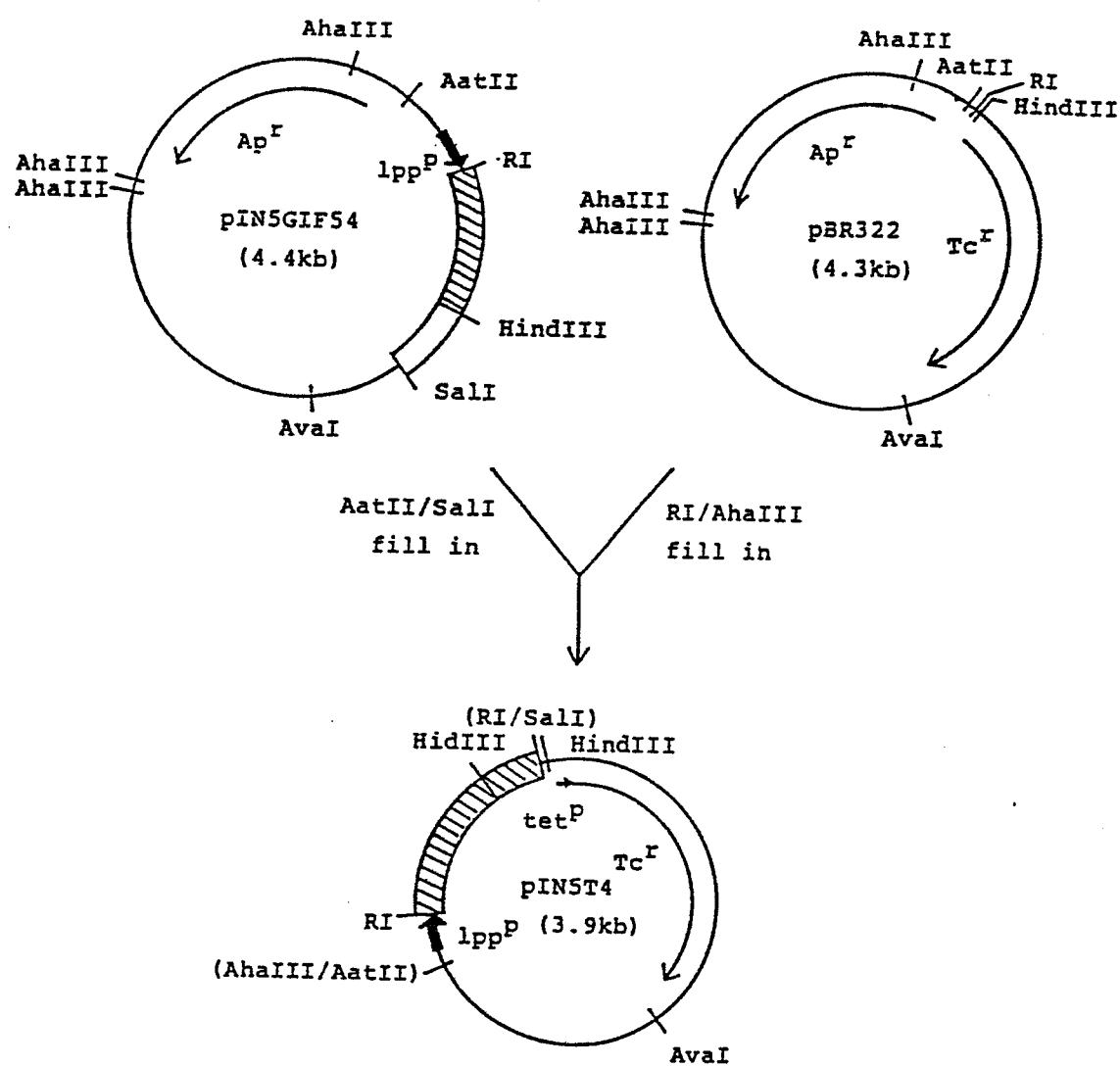
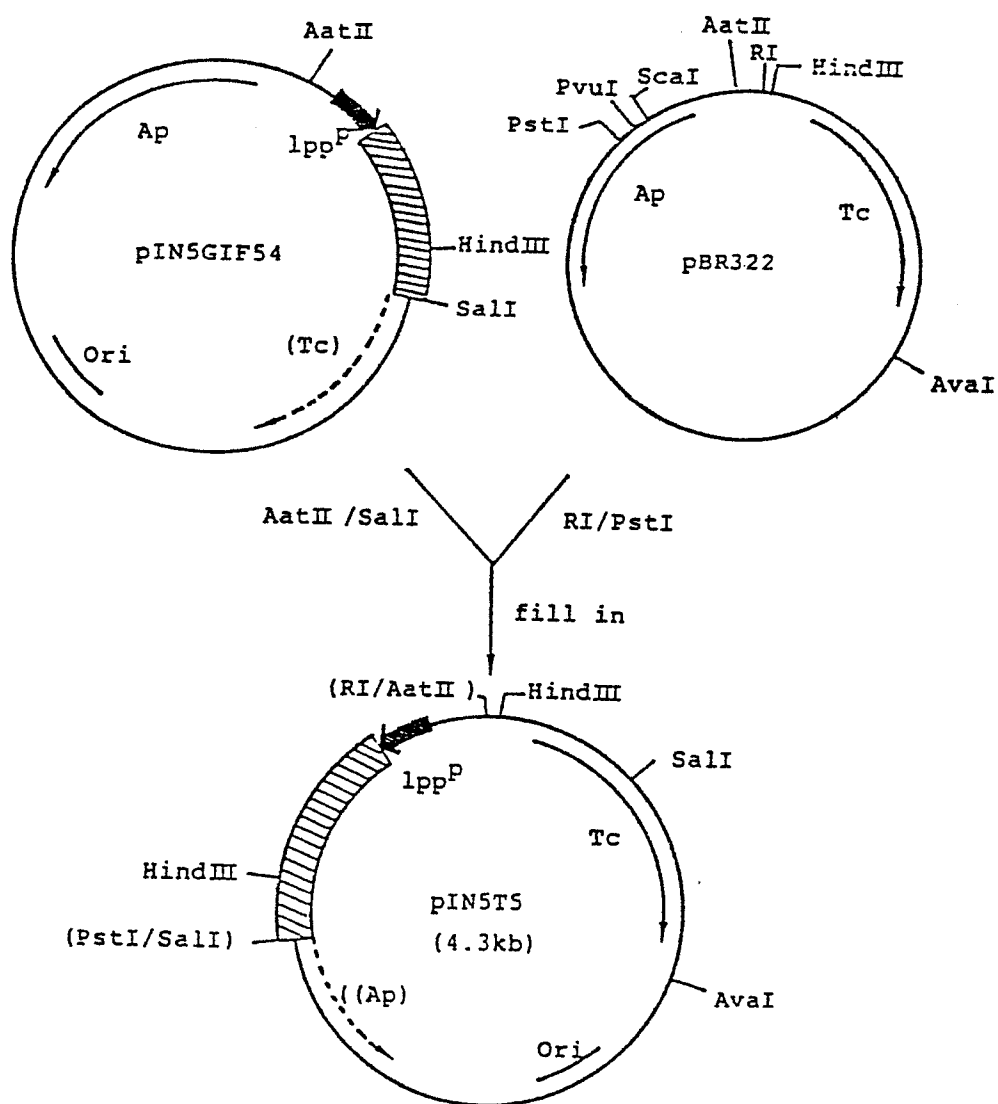


FIG. 8





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EUROPEAN SEARCH REPORT

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Application number

EP 84304901.6

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,A	<u>EP - A2 - 0 055 942</u> (RESEARCH FOUNDATION) * Claims 1-5,8,9,17 * & JP-Kokai-no. 140,800/1982 --	1,3,4,6	C 12 N 15/00 //C 12 R 1:19
P,A	<u>EP - A2 - 0 094 797</u> (RESEARCH FOUNDATION) * Claims 1-3,15,18 * --	1,3,4,6	
P,A	<u>EP - A1 - 0 095 361</u> (ELI LILLY) * Claims 1,3-5 * --	1,6	
A	<u>EP - A2 - 0 077 569</u> (TAMURA) * Claims 1,3,4,6; page 5, lines 10-15 * --	1,6	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
D,A	NATURE, vol. 287, no. 5579, September 18, 1980, New York/London D. DERYNCK et al. "Expression of human fibroblast interferon gene in Escherichia coli" pages 193-197 * Page 194 * --	1	C 12 N
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 24-10-1984	Examiner FARNIOK
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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Application number

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			TECHNICAL FIELDS SEARCHED (Int. Cl.) ☒ 4